



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07F 9/14, C07K 1/00, 1/04, C07F 9/12		A1	(11) International Publication Number: WO 98/12201
			(43) International Publication Date: 26 March 1998 (26.03.98)
(21) International Application Number: PCT/GB97/02592 (22) International Filing Date: 23 September 1997 (23.09.97) (30) Priority Data: 9619768.6 23 September 1996 (23.09.96) GB (71) Applicant (for all designated States except US): THE UNIVERSITY COURT OF THE UNIVERSITY OF ST. ANDREWS [GB/GB]; 66 North Street, St. Andrews, Fife KY16 9AH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): GANI, David [GB/GB]; Bois Feuris, Crail Road, St. Andrews, Fife KY16 8AP (GB). HILLIER, Mark [GB/GB]; 133B South Street, St. Andrews KY16 9UN (GB). HORMOZDIARI, Pantea [IR/GB]; 19 All Saints Court, Didcot, Oxon OX11 7NG (GB). (74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: PHOSPHORYLATING REAGENTS			
(57) Abstract			
<p>There is provided a phosphorylating reagent for phosphorylation of amino acids or compounds formed therefrom. The phosphorylating reagent is of utility in solution or solid-phase chemistry, and particularly for the solid-phase synthesis of phosphorylated peptides and combinational libraries of phosphorylated organic compounds. Also provided for is a method of phosphorylating oxygen, nitrogen and sulphur nucleophides, for example amino acid and peptides.</p>			

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1 **Phosphorylating Reagents**

2

3 This invention describes novel phosphorylating reagents
4 and the use thereof for solution or solid-phase
5 chemistry and their particular use for the solid-phase
6 synthesis of phosphorylated peptides and combinatorial
7 libraries of phosphorylated organic compounds.

8

9 The reversible phosphorylation of proteins on serine,
10 threonine and tyrosine residues, as catalysed by
11 protein kinases and phosphatases, is the principal
12 mechanism by which eukaryotic cells (the cells of
13 multicellular organisms) respond to external stimuli^{1,2}.
14 Three groups of enzymes referred to collectively as the
15 protein phosphatases (these enzymes hydrolyse the
16 phosphoryl group of a phosphoprotein) are responsible
17 for the dephosphorylation of the phosphoproteins. One
18 group known as the serine-threonine protein
19 phosphatases are collectively responsible for the
20 dephosphorylation of certain phosphorylated serine or
21 threonine residues within phosphoproteins (see Fig. 1A)
22 and several different types exist (e.g. PP1, PP2A, PP2B
23 and PP2C) most of which appear to be associated with
24 regulatory proteins. A second group are referred to as
25 the protein tyrosine phosphatases and these enzymes

1 hydrolytically remove the phosphoryl group from certain
2 phosphotyrosine residues within phosphoproteins, Fig.
3 1B. The third group of enzymes is responsible for
4 removing the phosphoryl group from phosphohistidine
5 residues within phosphoproteins, Fig. 1C.

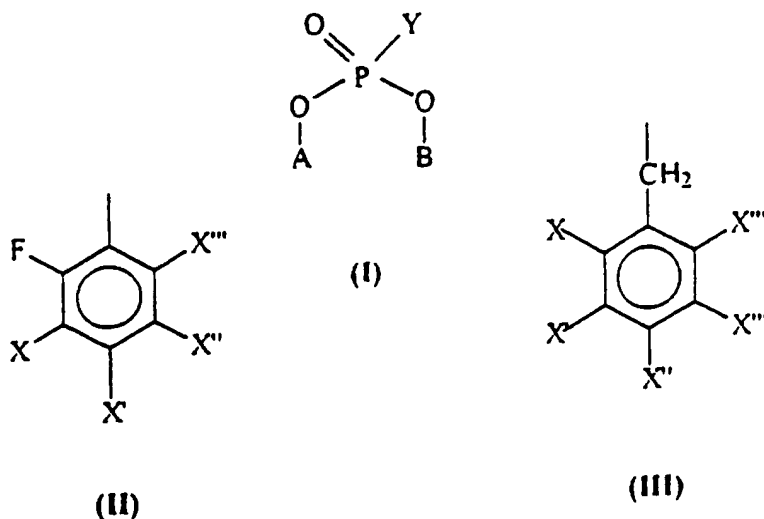
6
7 Structure-activity studies for the phosphorylated
8 peptide substrates of protein phosphatases have been
9 limited by the availability of structurally diverse
10 substrates because, to date, almost all of these have
11 been prepared by enzymic phosphorylation using
12 adenosine triphosphate (ATP) and appropriate protein
13 kinase enzymes which are specific for certain
14 sequences³. The specific nature of these enzymes
15 restricts the scope of these studies in addition to the
16 extra complications of separating the products of the
17 reaction e.g. separating the phosphorylated peptide
18 from adenosine monophosphate (AMP). Moreover, non-
19 enzymic syntheses of phosphopeptides, in particular
20 phosphothreonine peptides is severely hampered by β -
21 elimination of phosphoric acid diester which occurs in
22 synthetic intermediates to give the corresponding
23 dehydroamino acid moieties⁴⁻⁶. Phosphothreonine peptide
24 syntheses typically employ large excesses of highly
25 electrophilic phosphorus (III) reagents to introduce
26 phosphorus into the preformed peptide and then an
27 oxidant (e.g. tertiary-butyl hydroperoxide) is required
28 to convert the phosphite triester to the phosphate
29 triester prior to deprotection of the ester groups^{4,5}.
30 While the peptide exists as its phosphate triester, it
31 is particularly vulnerable to β -elimination, which is
32 undesirable.

33
34 The existing methods for avoiding β -elimination in the
35 synthesis of phosphoserine and phosphothreonine
36 peptides involve introducing each of the phosphorylated

1 amino acid residues as their protected phosphate
 2 diester monoanions⁶. These are however tedious to
 3 prepare.

4
 5 It is an object of the present invention to provide a
 6 phosphorylating agent that would be electrophilic
 7 enough to react directly and rapidly with primary and
 8 secondary alcohol groups within resin-bound peptides.
 9 Such agents would obviate the need for an oxidant, and
 10 could possess labile phosphate ester protecting groups
 11 that would be compatible with solid-phase peptide
 12 synthesis.

13
 14 This invention provides an electrophilic
 15 phosphorylating reagent for amino acids and/or peptide
 16 sequences thereof comprising a compound as represented
 17 by formula (I):



31 wherein: A is a substituted aromatic group which is
 32 represented by formula (II) e.g. a fluorophenyl or A is
 33 an acid cleavable functionality such as a benzyl or
 34 substituted benzyl group represented by formula (III);
 35 B is a substituted aromatic group represented by
 36 formula (II) e.g. a fluorophenyl group, but not a

1 benzyl or a substituted benzyl group;
2 each X, X', X'', X''' and X'''' are individually H or F
3 atoms or any suitable moiety;
4 Y is any halogen or leaving group.

5
6 The leaving group Y is the group which does not contain
7 the phosphorous atom following cleavage of compound I;
8 for example Y can be Cl, Br, I, -NRR'R'' (as a quaternary
9 ammonium salt), -OR, -SR (wherein each group R, R' or R''
10 is any group which does not affect the lability of the
11 leaving group Y, especially under acidic and/or basic
12 conditions, for example R, R' or R'' can individually be
13 -H, -CH₃, -C₂H₅, -C₆H₅, -C(O)-C₁₋₁₂ or -CH=C(OH)-C₁₋₁₂).

14
15 The reference to "any suitable moiety" with regard to
16 groups X, X', X'', X''' and X'''' refers to any atom or
17 group thereof which does not affect the lability of the
18 compounds represented by formulae II or III.

19
20 In one embodiment, the reagent is bis(tetrafluorophenyl)
21 chlorophosphate; and in this embodiment the preferred
22 reagent is bis (2, 3, 5, 6-tetrafluorophenyl)
23 chlorophosphate.

24
25 In a further embodiment, the reagent is a benzyl,
26 fluorophenyl halophosphate; and in this embodiment the
27 preferred reagent is a benzyl, polyfluorophenyl
28 chlorophosphate.

29
30 It can be seen that reagents represented by the formula
31 (I) have a fluorine in at least one *ortho* position on at
32 least one of the aromatic rings wherein the remaining
33 positions X, X', X'' and X''' on (II) and X,

34
35 X', X'', X''' and X'''' on (III) can each be -H or -F
36 atoms or any suitable moiety in any permutation.

1
2 Additionally, the -H atom or -F atom or suitable moiety
3 may be in the presence or absence of one or more
4 similar or dissimilar other ring substituents.

5
6 A further embodiment has a halogen or other leaving
7 group attached to the phosphorus atom of reagent (I) at
8 Y, where the leaving group can be one of -OR, -NRR',
9 -NRR'R'' or -SR, wherein R, R' and R'' can be any
10 suitable moiety.

11
12 The invention further provides a method for the
13 phosphorylation of oxygen, nitrogen or sulphur
14 nucleophiles of amino acids and/or peptides wherein the
15 nucleophile is treated with an excess of a reagent of
16 general formula (I) followed by hydrolysis of the
17 product.

18
19 Preferably the hydrolysis reagent is trifluoroacetic
20 acid.

21
22 The oxygen nucleophile may be part of a primary or
23 secondary alcohol, phenol, carboxylate or enolate
24 group.

25
26 The amino acids may be present as single species or in
27 combination within or outwith the same molecule, as in
28 peptide sequences.

29
30 Suitably, the amino acid(s) may be tyrosine, serine and
31 threonine.

32
33 In one particular embodiment of the invention, the
34 amino acid is present as a resin bound moiety.

35
36 In further embodiments of the invention, the

1 phosphorylation method may be utilised in solid, liquid
2 or gel phase.

3

4 The method is of considerable potential in the solid-
5 phase synthesis of a whole range of organic phosphates
6 from primary and secondary alcohols and phenols and is
7 completely compatible with combinatorial and
8 permutational organic synthesis.

9

10 In the area of peptide chemistry the method offers very
11 significant advantages over the previously used two
12 step phosphitylation-oxidation strategies,
13 furthermore, the use of bis-(pentafluorophenyl)
14 chlorophosphate (11) is of particular utility in the
15 preparation of peptides containing two or more
16 phosphorylated residues via a "global phosphorylation"
17 strategy which involves introducing all of the
18 phosphoryl groups in one step after the synthesis of
19 the required peptide. The same is true for the
20 introduction of more than one phosphoryl group into
21 other organic molecules which contain more than one
22 alcohol and/or phenol group.

23

24 The examples illustrate that primary alcohols,
25 secondary alcohols and phenols whether present as
26 single species, or in combination within or outwith the
27 same molecule, are efficiently phosphorylated by the
28 polyfluoroaromatic chlorophosphate reagents. Other
29 oxygen nucleophiles, for example, carboxylate and
30 enolate, and other nucleophiles, for example, those
31 derived from nitrogen and sulphur are also expected to
32 react with similar efficiency with the reagent.

33

34 The examples herein relate to the phosphorylation
35 reaction by bis-(pentafluorophenyl chlorophosphate (11)
36 and other polyfluoroaromatic halophosphates shown by

1 general formula I, where any, some or all X groups is H
2 and/or F or other suitable moiety in any permutation
3 whether in the presence or absence of one or more
4 similar or dissimilar other ring substituents; (Y is a
5 halogen or other leaving group) which should effect a
6 similar facile phosphorylation. Furthermore,
7 triesters, derived from oxygen nucleophiles, or any
8 other phosphorylated derivative containing the
9 polyfluoroaromatic phosphate diester protection;
10 wherein Y = -OR, -NRR', -NRR'R'', -SR, (where each group
11 R, R' or R'' can be any suitable moiety as defined
12 above) should be more labile to deprotection under
13 acidic conditions (and/or under basic conditions) than
14 the corresponding bis-phenyl phosphate diester
15 protection.

16

17 This method provides higher yields of phosphorylated
18 product of high quality with less or no wasteful side
19 reactions.

20

21 This invention is further described in a non-limiting
22 manner by reference to the following examples and
23 accompanying figures wherein:

24

25 Fig. 1a Illustrates the enzymatic dephosphorylation
26 of a phosphorylated threonine (or serine)
27 residue.

28

29 Fig. 1b Illustrates the enzymatic dephosphorylation
30 of a phosphorylated tyrosine residue.

31

32 Fig. 1c Illustrates the enzymatic dephosphorylation
33 of a phosphorylated histidine residue.

34

35 Fig. 2: Illustrates reaction schemes 1A & 1B.
36 Reagents and Conditions: i) 20%

1 piperidine/DMF; ii) 5% (CH₃CO)₂O/DMF; iii)
2 DMAP, TEA, PO(OPh)₂Cl, DCM, 20°C; iv) 82.5%
3 TFA: 5% phenol: 5% H₂O: 5% thioanisole; 2.5%
4 EDTA (reagent K), 80%; v) LiOH (aq),
5 EtOH/CH₃CN; vi) DMAP, TEA, PO(OPhF₅)₂Cl, DCM,
6 20°C; vii) Dowex Cl, 60%.

7
8 Fig. 3a: Shows the structure of bis(pentafluorophenyl)
9 chlorophosphate (11).

10
11 Fig. 3b: Shows the structure of the
12 bis(pentafluorophenyl) phosphate derivative
13 of cyclohexanol (12).

14
15 Fig. 4: Shows the structure of pentafluorobenzyl-
16 pentafluorophenyl chlorophosphate (13).

17
18 Fig. 5: Illustrates reaction scheme 2. Reagents and
19 Conditions: i) 1.01 eq *N*-Chlorosuccinimide,
20 toluene, 2hr, rt; ii) NaH, C₆F₅OH, THF, 1hr,
21 rt; iii) a) NaI, acetone, Δ, 15 mins. b)
22 HCl_(aq); iv) PCl₅, DCM.

23
24 Fig. 6a: Shows the structure of the benzyl
25 pentafluorophenyl derivative of cyclohexanol
26 (18).

27
28 Fig. 6b: Shows the structure of the benzyl
29 pentafluorophenyl derivative of *N*-α-^tBoc-
30 tyrosine methyl ester (19).

31
32 Fig. 6c: Shows the structure of the phosphopeptide
33 Asp-Ala-Asp-Glu-Tyr(OPO₃H₂)-Leu (23).

34
35 Fig. 7: Illustrates reaction scheme 3. Reagents and
36 Conditions: i) 20% piperidine/DMF; ii) DMAP,

1 TEA, PO(OCH₂Ph)(OPhF₃), DCM, 20°C; iii) NaOH
2 (aq), DMSO; iv) 90% TFA, 5% H₂O, 5% Et₃SiH.
3

4 Example 1

5
6 Diphenyl chlorophosphate had been successfully employed
7 to phosphorylate the secondary alcohol groups of myo-
8 inositol and its analogues'. Using an N-acetyl (Ac)
9 capped analogue of a known consensus sequence for a
10 PP2A substrate as the target, AcNH-Arg-Arg-Ala-
11 Thr(PO₃H₂)-Val-Ala-OH (1), a series of solid-phases
12 phosphorylation reactions were examined. Accordingly,
13 using Wang resin, standard Fmoc chemistry with PyBOP
14 activation, and arginine residue precursors containing
15 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)
16 protected guanidino groups, the peptide Fmoc-NH-Arg-
17 Arg-Ala-Thr-Val-Ala-O-Wang (2) was prepared. The N-
18 terminal Fmoc group was removed with 20% piperidine in
19 DMF and the free amino group was capped with 5% acetic
20 anhydride in DMF to give compound (3). Treatment of
21 the resin-bound peptide (3) with diphenyl
22 chlorophosphate gave some of the required diphenyl
23 threonine phosphate triester (4), and under optimised
24 conditions (repeated treatments with 20 equivalents of
25 diphenyl chlorophosphate in the presence of DMAP and
26 TEA for 6-8 hours at ambient temperature) essentially
27 quantitative conversion to the triester (4) could be
28 achieved, as determined by NMR-spectroscopic analysis
29 of the products after cleavage from the resin Fig. 2,
30 Scheme 1A.
31
32 ¹H-, ¹³C- and ³¹P-NMR spectra showed the expected
33 signals, chemical shift changes and P-C and P-H
34 couplings for the required triester (5). All attempts
35 to hydrolyse the pure triester (5) under mild basic
36 conditions resulted in the formation of significant

1 quantities of the β -elimination product,
2 dehydrobutyrine peptide (6), as judged by ^1H - and ^{31}P -
3 NMR spectroscopy.

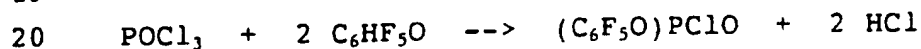
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5 Example 2

6

7 In order to increase the electrophilicity at phosphorus
8 in the phosphorylating species (to decrease reaction
9 times) and also in the required peptide phosphate
10 triester (to facilitate deprotection), the preparation
11 and use of bis-(pentafluorophenyl) chlorophosphate (11)
12 was investigated. The reagent was prepared by treating
13 phosphorus oxychloride (7) with 1.8 equivalents of
14 pentafluorophenol (8) at 140°C for 16-24 hours and was
15 purified by removing the unreacted starting materials
16 by distillation. The resulting reagent (11) was 85-90%
17 pure as judged by ^{19}F - and ^{31}P -NMR spectroscopy and could
18 be further purified by fractional distillation.

19



21

22 Example 3

23

24 In model reactions using cyclohexanol, the bis-
25 (pentafluorophenyl) chlorophosphate (11) reacted at
26 least 30-fold more rapidly than diphenyl
27 chlorophosphate to give the required triester (12)
28 which was fully characterised. Note that the bis-
29 (2,3,5,6-tetrafluorophenyl) chlorophosphate analogue of
30 reagent (11), which was more useful for mechanistic
31 studies and for product characterisation (due to the
32 presence of an integratable proton resonance in ^1H -NMR
33 spectra), behaved similarly in effecting rapid
34 phosphorylation.

35

36

1 Example 4

2

3 Treatment of the Pmc protected resin-bound peptide, Ac-
4 NH-Arg-Arg-Ala-Thr-Ala-Val-Ala-O-Wang(3), with 10
5 equivalents of bis-(pentafluorophenyl) chlorophosphate
6 under optimised conditions gave the resin-bound
7 phosphate triester (9) in excellent yield, Scheme 2B.
8 Immediate deprotection of the two Pmc groups, the two
9 pentafluorophenyl groups, and simultaneous cleavage
10 from the resin occurred upon treatment with aqueous
11 trifluoroacetic acid solutions to give the almost pure
12 N-capped phosphorylated threonine peptide (10) in
13 essentially quantitative conversion. There was no
14 evidence whatsoever for β -elimination products and
15 purification on Dowex 1 chloride (Trade Mark) gave the
16 pure phosphopeptide (10) in 60% overall yield (over 14
17 solid-phase steps). This material was fully
18 characterised and served as a substrate for protein
19 phosphatase λ as judged by directly monitoring the
20 course of phosphopeptide hydrolysis by $^1\text{H-NMR}$
21 spectroscopy.

22

23 Example 5

24

25 Other peptides containing serine residues or tyrosine
26 residues were also successfully phosphorylated with
27 bis-(pentafluorophenyl) chlorophosphate (11) using
28 similar protocols.

29

30 Example 6

31

32 Merrifield resin bound inositol analogues, connected by
33 ether linkages which are stable to trifluoroacetic
34 acid, were successfully phosphorylated on secondary
35 alcohol moieties by bis-(pentafluorophenyl)
36 chlorophosphate (11) using similar protocols.

1 Treatment with aqueous trifluoroacetic acid resulted in
2 the deprotection of the pentafluorophenyl groups to
3 give resin bound inositol monophosphate analogues.
4 (6p (121.41 MHz, C₆²H₆): -10.443).

5
6 Example 7

7
8 In both solution and solid phase phosphorylations of
9 phenols it was noted that, whilst the actual
10 phosphorylation reaction with bis-(pentafluorophenyl)
11 chlorophosphate (11) was facile, complete removal of
12 the pentafluorophenyl groups was difficult. In each
13 case, the first pentafluorophenyl group could be
14 removed easily in the presence of trifluoroacetic acid
15 solution, but the second pentafluorophenyl group could
16 not. Therefore, since it appeared that the acidity of
17 the partially deprotected phosphoric acid derivative
18 was too high for protonation by the trifluoroacetic
19 acid solution, modified reagents were designed, [for
20 example preferably formula I, where II is a substituted
21 phenyl group (where X, X', X'', X''' are H or F atoms
22 or any suitable moiety), III is a benzyl or substituted
23 benzyl group (where X, X', X'', X''', X'''' are H or F
24 atoms or any suitable moiety) but not a phenyl or
25 substituted phenyl group, and Y is any halogen. It was
26 expected that the phenyl or substituted phenyl group
27 (derived from the reagent) of the intermediate triester
28 (phosphorylated alcohol or phenol) would be removed in
29 a facile manner by base catalysed hydrolysis, and that
30 the benzyl or substituted benzyl group could be removed
31 in a facile manner by acid catalysed hydrolysis,
32 preferably in the presence of trifluoroacetic acid,
33 which would be compatible with other solid state
34 synthesis protocols.

35

36 To prepare such substituted phenyl substituted benzyl

1 halophosphates, a model synthetic protocol was
2 developed using benzyl pentafluorophenyl chlorophosphate
3 as the target (Scheme 2).

4
5 Treatment of dibenzyl phosphite (14) with *N*-
6 chlorosuccinimide in toluene⁸, followed by reaction with
7 sodium pentafluorophenolate (formed by the reaction
8 between sodium hydride and pentafluorophenol in THF)
9 resulted in the formation of dibenzyl pentafluorophenyl
10 phosphate triester (15). ¹H, ¹³C, ¹⁹F and ³¹P-NMR spectra
11 showed the expected chemical shift changes and P-C and
12 P-H coupling constants consistent with those expected
13 for the required triester. $\delta_H(300 \text{ MHz, C}^2\text{HCl}_3)$: 5.21 (d,
14 J_{PH} 8.7, CH₂OP), $\delta_C(75.4 \text{ MHz, C}^2\text{HCl}_3)$: 70.88 (d, CH₂OP, J_{PC}
15 6.5), $\delta_P(121.41 \text{ MHz, C}^2\text{HCl}_3)$: -5.44, and the correct
16 mass ion (m/z (CI+ mode) 444, M⁺ molecular ion).

17
18 Reaction of the triester (15) with 1 equivalent of
19 anhydrous sodium iodide in refluxing acetone for 15
20 minutes gave a white solid, which upon cooling was
21 isolated by filtration, then dissolved in water and
22 treated with aqueous hydrochloric acid.⁹ The resulting
23 precipitate of benzyl pentafluorophenyl phosphoric acid
24 diester (16) was isolated in essentially quantitative
25 yield from the dibenzyl pentafluorophenyl phosphate
26 triester (15). ¹H, ¹³C, ¹⁹F and ³¹P-NMR spectra showed
27 the expected chemical shift changes and P-C and P-H
28 coupling constants for the required diester. $\delta_H(300$
29 $\text{MHz, C}^2\text{HCl}_3)$: 5.20 (2H, d, J_{PH} 8.4, CH₂OP), $\delta_C(75.4 \text{ MHz,$
30 $\text{C}^2\text{HCl}_3)$: 70.92 (d, CH₂OP, J_{PC} 5.4), $\delta_P(121.41 \text{ MHz, C}^2\text{HCl}_3)$:
31 -4.66. Mass spectrometry confirmed the desired product
32 had been obtained (m/z (EI+ mode): 354 (M⁺ molecular
33 ion)). Reaction of benzyl pentafluorophenyl phosphoric
34 acid diester (16) with an excess of PCl₅ in
35 dichloromethane followed by removal of the solvent at
36 reduced pressure (20mm/Hg) and separation of the by-

1 products by distillation at 0.1 mm Hg/30-40°C afforded
2 the reagent (17) in better than 75% purity as judged by
3 ^1H and ^{31}P -: 1R. δ_{H} (300 MHz, C^2HCl_3):5.38 (2H, d, J_{PH} 9.9,
4 CH_2OP), δ_{C} (75.4 MHz, C^2HCl_3):72.91 (d, CH_2OP , J_{PC} 7.54),
5 δ_{P} (121.41 MHz, C^2HCl_3): main peak at -2.39. Mass
6 spectrometric analysis also gave the expected data (m/z
7 (EI+): 372, 374 (Cl isotopes, M^+ molecular ion). The
8 reagent was found to be unstable at high temperatures
9 (50°C) and decomposed if heated for prolonged periods
10 above that temperature. The major contaminant
11 displayed 2 signals at -18.6 and -19.5 ppm in the ^{31}P
12 NMR spectrum of the product and corresponding signals
13 in the ^1H , ^{13}C and ^{19}F NMR spectra, consistent with the
14 expected properties of the bis-(benzyl)-bis-
15 (pentafluorophenyl) pyrophosphate. The mass spectrum
16 of the contaminant showed a molecular fragment (m/z
17 (CI+) 507, $[\text{M}-\text{OPhF}_5]^+$) consistent with the structure of
18 the pyrophosphate. Since this material would give
19 identical phosphorylated products to the
20 chlorophosphate, the crude reagent was used routinely
21 for solid phase phosphorylations.

22
23 Other benzyl phenyl chlorophosphates were prepared
24 using analogous methods.

25 Example 8

26
27
28 In model phosphorylation reactions in solution using
29 cyclohexanol, the benzyl pentafluorophenyl
30 chlorophosphate (17) reacted with cyclohexanol in the
31 presence of triethylamine in dichloromethane to give
32 the required triester (18). This was characterised by
33 ^1H , ^{13}C , ^{19}F and ^{31}P -NMR spectroscopy and gave the
34 expected data.

35
36

1 Example 9

2
3 In model phosphorylation reactions in solution using
4 N-^tBoc-(2S)-tyrosine methyl ester, the benzyl
5 pentafluorophenyl chlorophosphate (17) reacted with the
6 phenolic hydroxyl group in the presence of
7 triethylamine in dichloromethane to give the required
8 triester(19). This was characterised by ¹H, ¹³C, ¹⁹F and
9 ³¹P-NMR spectroscopy and mass spectrometry and gave the
10 expected data.

11
12 Example 10

13
14 In model solid state phosphorylation reactions,
15 treatment of the resin-bound peptide Fmoc-Val-Tyr-Leu-
16 O-Wang (20) with 10 equivalents of freshly prepared
17 benzyl pentafluorophenyl chlorophosphate (17) under
18 optimised conditions gave the resin bound phosphate
19 triester (21) in excellent yield, Scheme 3. Treatment
20 with 20% piperidine in DMF removed the N-terminal Fmoc
21 group. Subsequent treatment of the product with an
22 excess of 1 mol.dm⁻³ aqueous NaOH in DMSO followed by
23 washing and treatment with aqueous trifluoroacetic acid
24 resulted in deprotection of the pentafluorophenyl and
25 benzyl groups and cleavage of the resin C-terminal
26 ester linkage to give the phosphopeptide Val-
27 Tyr(OPO₃H₂)-Leu (23), δ_p (121.41 MHz, ²H₂O):-3.42.

28
29 Example 11

30
31 Treatment of the *tris*-tert-butyl ester protected resin
32 bound peptide Fmoc-NH-Asp(O^tBu)-Ala-Asp(O^tBu)-Glu(O^tBu)-
33 Tyr-Leu-O-Wang in a similar manner to that described in
34 Example 10 above afforded the almost pure hexapeptide
35 Asp-Ala-Asp-Glu-Tyr(OPO₃H₂)-Leu (24) which showed the
36 expected NMR spectroscopic data. This product

1 corresponds to the structure of the autophosphorylation
2 site of the epidermal growth factor receptor (EGFR)¹⁰ in
3 its phosphorylated form.

4

5 Example 12

6

7 Treatment of the resin bound and protected peptide Ac-
8 NH-Arg(Pmc)-Arg(Pmc)-Ala-Thr-Val-Ala-O-Wang (3) with 10
9 equivalents of benzyl pentafluorophenyl chlorophosphate
10 (17) under optimised conditions gave the benzyl
11 pentafluorophenyl peptide phosphate triester.

12 Treatment of the resulting triester overnight with an
13 excess of 1 mol.dm⁻³ aqueous NaOH in DMSO followed by
14 washing and subsequent treatment with aqueous
15 trifluoroacetic acid resulted in deprotection of the
16 two 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)
17 groups, the pentafluorophenyl and benzyl groups and
18 cleavage of the C-terminal resin ester moiety to give
19 the almost pure N-capped phosphohexapeptide (10).
20 Spectroscopic data showed this material to be identical
21 to that prepared in Example 4 above.

22

23 The serine analogue of (10) was prepared using a
24 similar protocol.

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1 Claims

2

- 3 1. An electrophilic phosphorylating reagent for amino
4 acids and/or peptide sequences thereof comprising
5 of a compound represented by formula (I) wherein:

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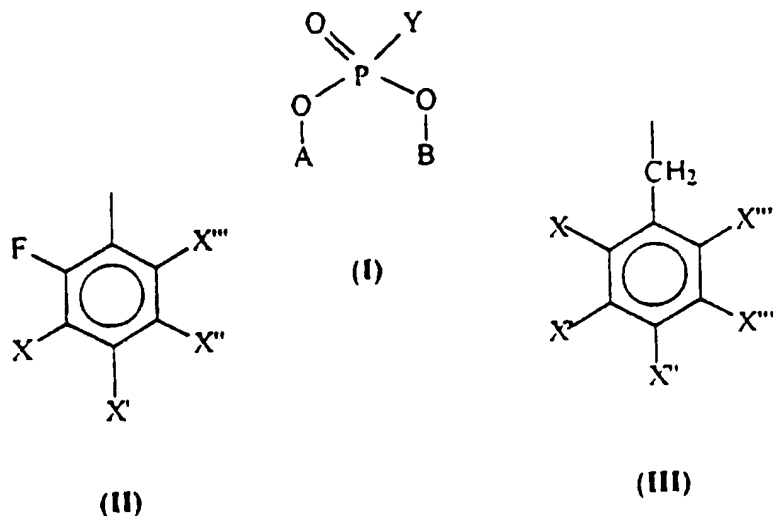
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A is a substituted aromatic group which is represented by formula (II) or A is an acid cleavable functionality such as a benzyl or substituted benzyl group represented by formula (III);

B is a substituted aromatic group represented by formula (II); X, X', X'', X''' and X'''' are each H or F atoms or any suitable moiety; Y is a halogen or leaving group.

2. A phosphorylating reagent as claimed in Claim 1, wherein at least one of the compounds represented by formulae (II) and (III) is fully substituted by fluorine.

3. A phosphorylating reagent as claimed in either of Claims 1 and 2 wherein group Y is a chlorine atom.

- 1 4. A phosphorylating reagent as claimed in any one of
2 Claims 1 to 3 which is bis(pentafluorophenyl)
3 chlorophosphate .
4
- 5 5. A phosphorylating reagent as claimed in Claim 3
6 which is bis (2, 3, 5, 6 - tetrafluorophenyl)
7 chlorophosphate.
8
- 9 6. A phosphorylating reagent as in any one of Claims
10 1 to 3 where the reagent is a benzyl, fluorophenyl
11 halophosphate.
12
- 13 7. A phosphorylating reagent as in claim 6 where the
14 reagent is a benzyl, polyfluorophenyl
15 chlorophosphate.
16
- 17 8. A method for the phosphorylation of oxygen,
18 nitrogen or sulphur nucleophiles of amino acids
19 and/or compounds comprising an amino acid-like
20 moiety wherein the nucleophile is treated with an
21 excess of a reagent of general formula (I) as
22 defined in Claim 1 followed by the hydrolysis of
23 the product.
24
- 25 9. A method as in claim 8 where the oxygen
26 nucleophile may be part of a primary or secondary
27 alcohol, phenol, carboxylate or enolate group.
28
- 29 10. A method as in either one of claims 8 and 9 where
30 the amino acids may be present as single species
31 or in combination within or outwith the same
32 molecule, as in peptide sequences.
33
- 34 11. A method as in claim 10 where the amino acid(s)
35 may be tyrosine, serine and threonine.
36

- 1 12. A method as in any one of claims 8 to 11 where the
2 amino acid and/or peptide is present as a resin
3 bound moiety.
4
- 5 13. A method as in any one of claims 8 to 12 where the
6 phosphorylation method may be utilised in solid,
7 liquid or gel phase.
14. A method as in any one of claims 8 to 13 where the
 hydrolysis reagent is trifluoroacetic acid.

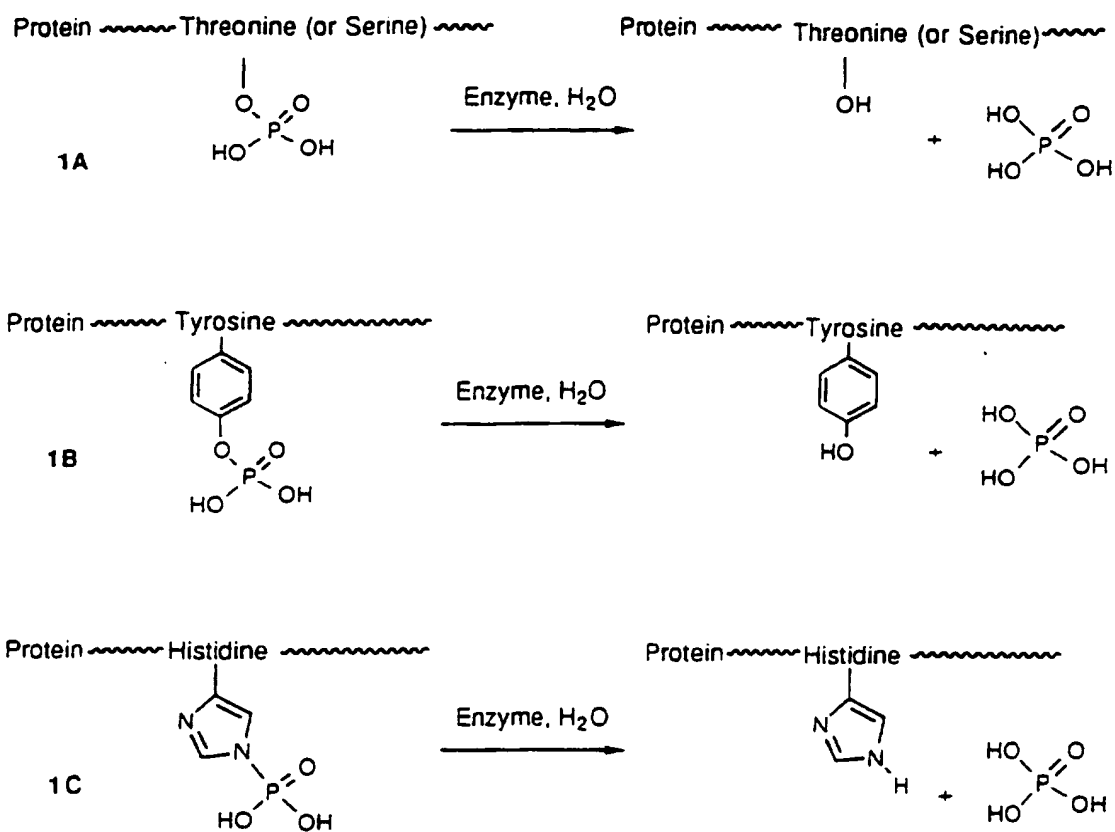


FIGURE 1

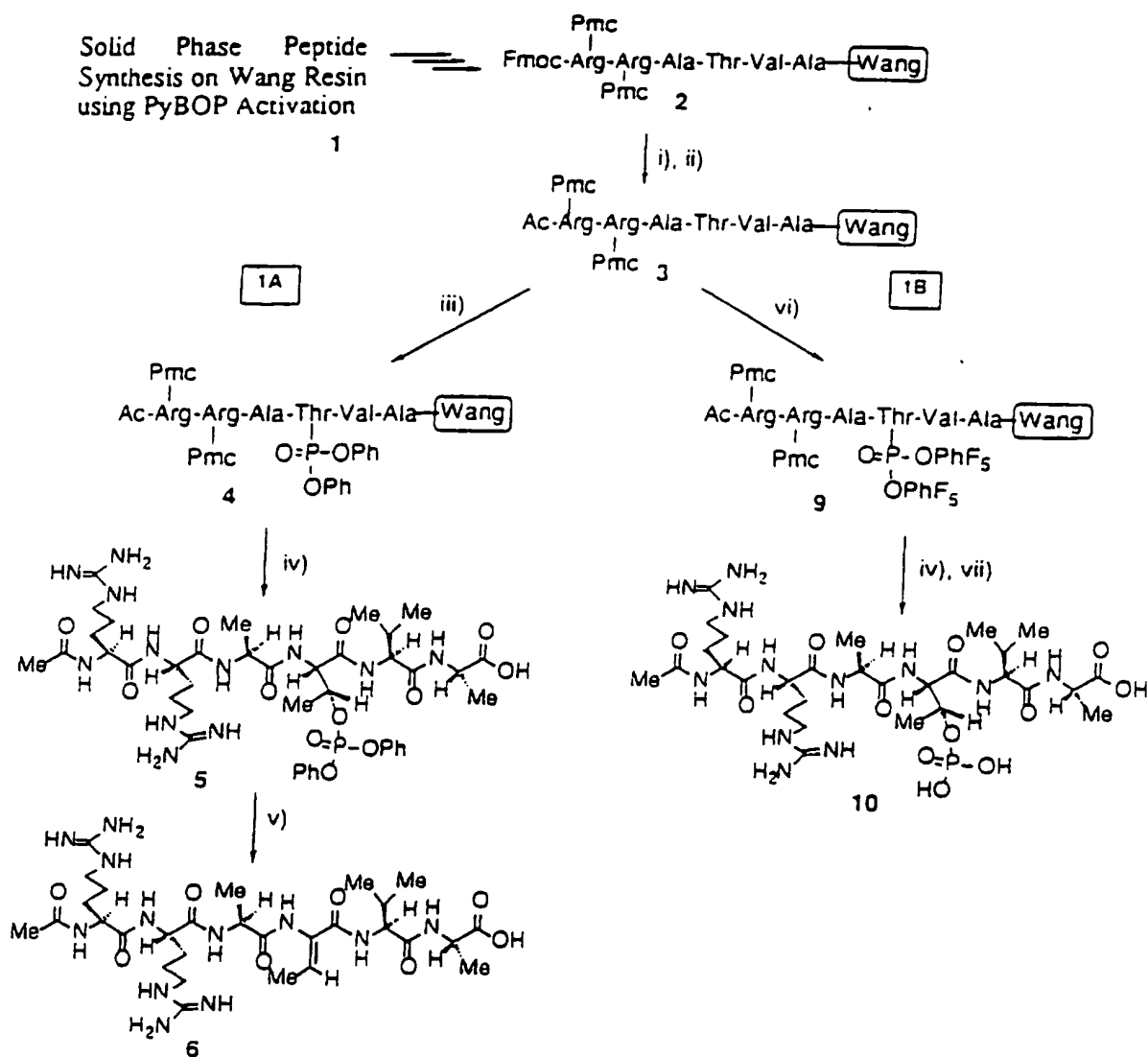


FIGURE 2

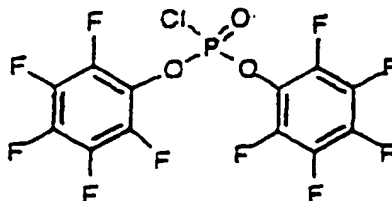


FIGURE 3A

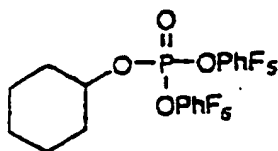
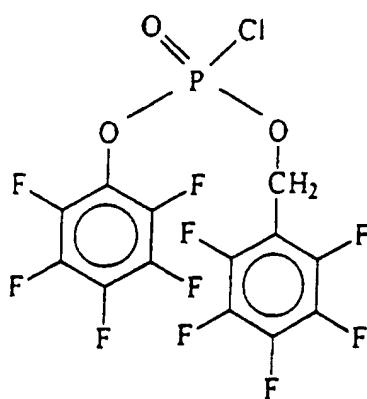


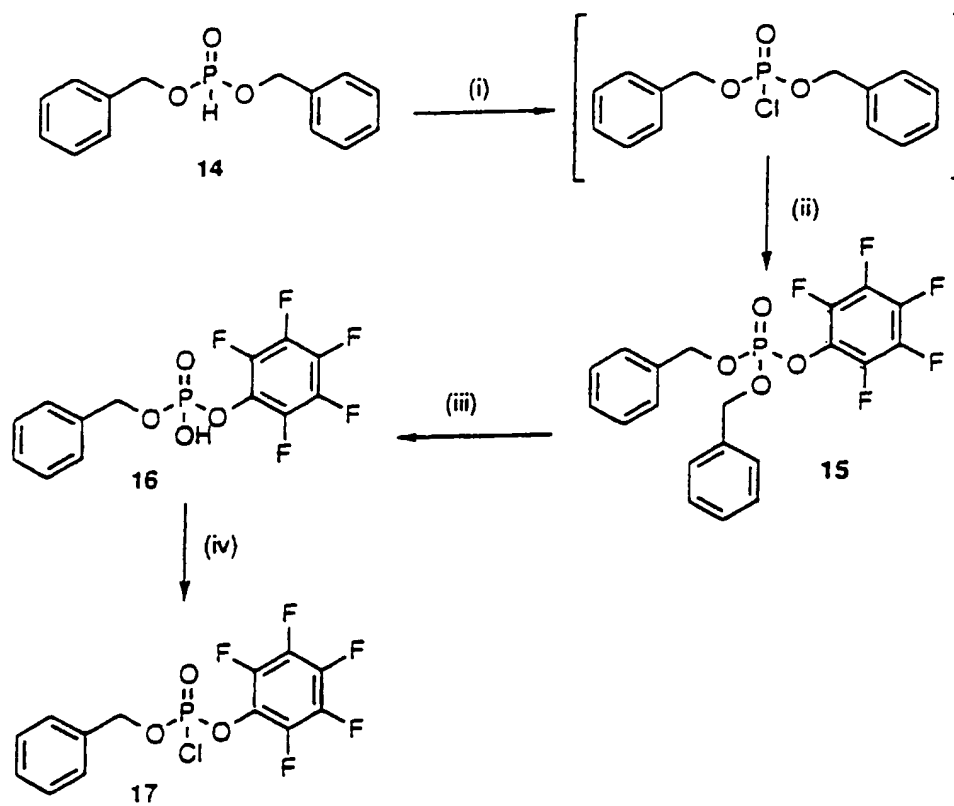
FIGURE 3B



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FIGURE 4

FIGURE 5



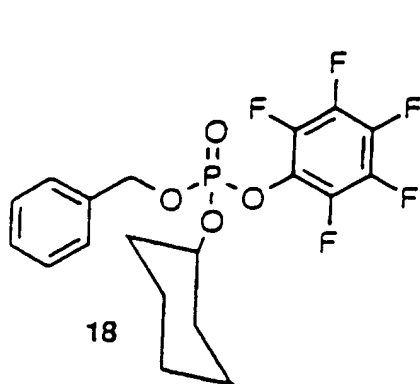


FIGURE 6A

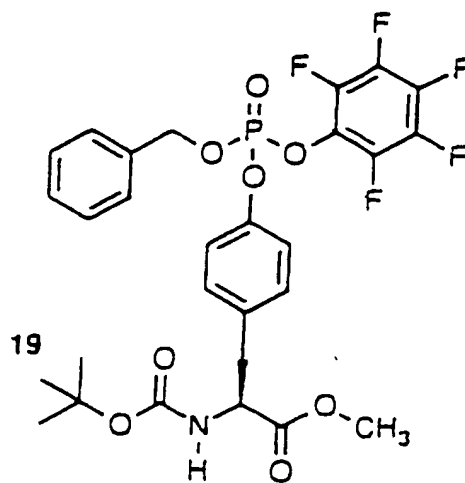


FIGURE 6B

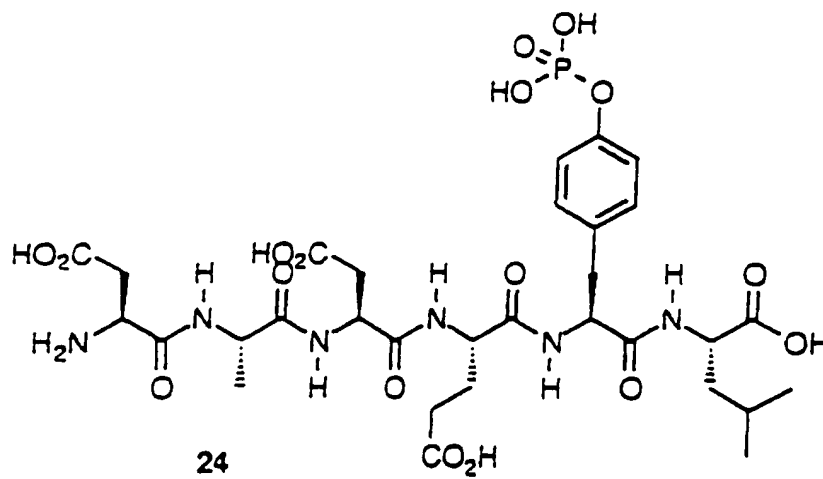
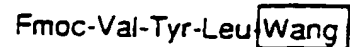
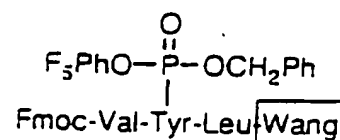
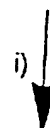


FIGURE 6C

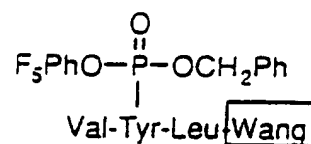
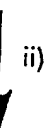
Solid Phase Peptide Synthesis
on Wang Resin using Pybop
Activation



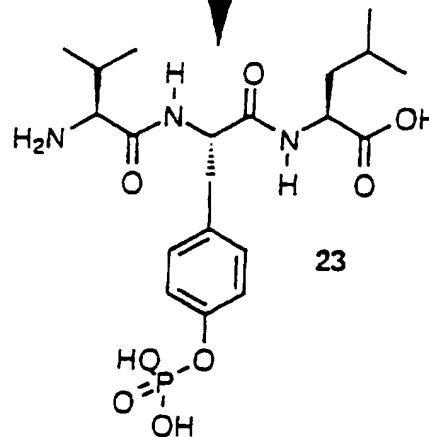
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FIGURE 7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/02592

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07F9/14 C07K1/00 C07K1/04 C07F9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07F C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 341 630 A (ROBERT H. BOSCHAN) 12 September 1967 see the whole document ---	1-4
X	US 3 341 631 A (CHRISTIAN A. SEIL) 12 September 1967 see the whole document ---	1,2
X	US 3 408 427 A (ROBERT H. BOSCHAN) 29 October 1968 see the whole document ---	1,3
Y	US 5 245 069 A (JAMES W. MCMANUS) 14 September 1993 see the whole document ---	1-14
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Date of the actual completion of the international search

17 November 1997

Date of mailing of the international search report

04/12/1997

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02592

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHULZ J ET AL: "Synthesis and properties of mechanism-based inhibitors and probes for inositol monophosphatase derived from 6-O-(2'-hydroxyethyl)-(1R,2R,4R,6R)-cyclohexane-1,2,4,6-tetraol" J. CHEM. SOC., CHEM. COMMUN. (JCCCAT,00224936);95; (22); PP.2353-6, THE UNIVERSITY, ST. ANDREWS;SCHOOL CHEMISTRY; FIFE; KY16 9ST; UK (GB), XP002047053 cited in the application see the whole document ---	1-14
Y	JAN HES: "Di(2-tert-butylphenyl) Phosphorochloridate. A new selective Phosphorylating agent." JOURNAL OF ORGANIC CHEMISTRY., vol. 39, no. 25, 1974, EASTON US, pages 3767-3769, XP002047054 see the whole document ---	1-14
P,X	HORMOZDIARI P ET AL: "Highly efficient solid-phase phosphopeptide synthesis using bis(polyfluorophenyl) chlorophosphates: preparation of serine-threonine protein phosphatase substrates" TETRAHEDRON LETT. (TELEAY,00404039);96; VOL.37 (45); PP.8227-8230, THE UNIVERSITY;SCH. CHEM.; ST. ANDREWS, FIFE; KY16 9ST; UK (GB), XP002047055 see the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 97/02592

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 3341631 A 12-09-67 NONE

US 3408427 A 29-10-68 NONE

US 5245069 A 14-09-93 NONE